PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12M 1/00, 1/12, C12Q 1/00, 1/06, 1/24, 1/68, G01N 33/543, 33/569

A1

(11) International Publication Number: WO 99/45094

(43) International Publication Date: 10 September 1999 (10.09.99)

(21) International Application Number: PCT/US99/04415

(22) International Filing Date: 1 March 1999 (01.03.99)

(30) Priority Data:
09/032,964 2 March 1998 (02.03.98) US
09/094,256 9 June 1998 (09.06.98) US

(71) Applicant: COMPUCYTE CORP. [US/US]; 12 Emily Street, Cambridge, MA 02139 (US).

(72) Inventor: KAMENTSKY, Louis, A.; 180 Beacon Street, Boston, MA 02116 (US).

(74) Agent: NISSENBAUM, Israel; Graham & James LLP, 21st floor, 885 Third Avenue, New York, NY 10022 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: SELECTIVE CELL ANALYSIS

(57) Abstract

A method and device for the laser scanning analysis of a selected small portion of specimen cells in a stationary and very localized area of a specimen slide, which cells are primarily specifically selected to be of scanning interest. The specimen may be pretreated with an adhering medium specifically tailored to characteristics of the selected cells whereby specific cells are made adherent thereby. The specimen slide is then locally provided with a co–acting adhering material or adhering device, or filtering device in a non–impeding gate position, whereby a statistically significant number of selected cells do not reach or are adhered to the gate position and remaining non–selected cells are removed from the gate position. It is the gate position which is then scanned by the laser scanning cytometer with increased efficiency. The adhering materials are either coated onto the surface of the gating area or are provided with magnetically susceptible particles. In the latter embodiment, a localized magnet is placed adjacent the gating area to adhere magnetized selected cells to the gating area. The cells of interest may be a cell class or set having a subset (e.g., white blood cells and monocytes), with characteristics of the subset being of analytic interest. Accordingly, initial reagents are used for the adhering and additional reagents are used for marking the subset and analyzing the desired characteristics.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

SELECTIVE CELL ANALYSIS

FIELD OF THE INVENTION

This invention relates to devices and methods used in selectively separating cells of varying types and characteristics from each other for analysis and particularly to such methods and devices used with laser scanning cytometers.

BACKGROUND OF THE INVENTION

Many important cell based assays for various medical conditions have been developed through the use of flow cytometry. Flow cytometry instruments measure multiple optical properties of cells as they are made to flow in a cuvette in single file through a light beam, often a laser. As the cell interacts with the light, both fluorescent and scatter emissions are given off that can be measured by photo sensors. If the cells are appropriately reacted prior to measurement, with fluorescent dyes that bind to specific cellular constituents, the amount of fluorescence measured can be directly related to each of a specific cellular constituent, such as DNA, RNA or specific proteins. These dyes can be conjugated by well established techniques to antibodies which can in turn bind to specific proteins in the cell. The resulting fluorescence, measured by a flow cytometer's sensors, can be used to quantify the amount of a specific protein per cell or to differentially count one or more types of cell in a heterogeneous cell population such as blood. Additionally, fluorescence dyes can be attached to specific nucleic acid sequences, which in turn, will bind to specific DNA or RNA sequences within the cell's nucleic acids to mark cells with specific genotypes.

This latter technique, because preparations are difficult to perform on cells in suspension (as required in flow cytometry), can be more advantageously be done by the more recently developed laser scanning cytometry (Described in U.S. Patent No. 5,107,422), currently a laser scanning cytometer is commercially available from CompuCyte Corporation, Cambridge, MA. under the trademark LSC®, which is designed for measuring

cells prepared and stationarily positioned on a microscope slide surface.

Although flow cytometry technology has been widely used in biomedical research, it has not been widely applied to clinical use, with the exceptions of diagnosis and monitoring of leukemia and AIDS. Laser scanning cytometry, with its stationary samples, is more appropriate for use in all other clinical settings. In laser scanning cytometry, fluorescence and scatter is measured in cells placed on a solid substrate such as a microscope slide. Prepared cells (as described above with respect to flow cytometry), are measured by moving a laser beam over the substrate and moving the substrate (without separate sample movement) under computer control, to find the cells on the substrate and to measure their fluorescence and scatter emissions. Data obtained by scanning cytometry has been shown to be equivalent to that of flow cytometry but with greater efficiencies of use and with less margin for operator error (see e.g. Kamentsky et al *Slide-Based Laser Scanning Cytometry*, Acta Cytologica 41:123-143 (1997)). In addition, laser scanning cytometry has been shown to have a number of benefits not available with use of flow cytometry.

First, heterogeneous populations of cells on a slide can be scanned, multiple properties of the cells measured, and the instrument under user direction can relocate cells with specific defined sets of properties for visual observation or photography or to combine data from multiple assays using the location of each cell as the data merge key.

Second, since each cell is scanned with many data elements, constituents of cells can be localized to cell compartments such as the nucleus or cytoplasm and tests such as *in-situ* hybridizations, in which fluorescent spots are counted, can be performed.

Third, since the cells are measured on the surface of a substrate such as a slide, certain assays such as *in-situ* hybridizations are possible because they are difficult to do on

suspended cells because cells do not remain intact they are lost due to the multiple centrifugations required.

It is also possible to perform complex procedural steps on the cells without separating the cells from the medium in which they are presently contained. This is presently done primarily by centrifugation to bring down the cells so that the supernatant can be poured off. For users of flow cytometry, heterogeneous populations of cells such as blood must be pretreated prior to analysis by separating specific cell types by centrifugations, columns, settling or lysis. The cells must then be reacted with reagents such as antibody conjugated fluorochromes, and the cells must be washed between and after these reactions using laboratory centrifuges. As a result, flow cytometry has not been adopted for many useful diagnostic and prognostic applications because the preparative procedures are complex, requiring highly trained personnel to perform and control the various protocols and run the instrument. Furthermore, for a number of applications, these tests must be performed in emergency rooms, in operating theaters, near neonatal units or in physician's offices where highly trained operators are not available and yet results are needed in minutes to determine patient treatment. Laser scanning cytometry obviates many of such problems.

There are however, limitations unique to the use of laser scanning cytometers. For example, an important characteristic of such use is that the time required to measure a specimen is directly proportional to the area scanned. Thus, if 10,000 cells are placed over a 10 millimeter square area it will take ten times as long to process the specimen than if it were placed in a one millimeter square area. Because of present computer speeds, data processing is so rapid that the time to process a specimen is primarily controlled and limited by the time it takes to scan the laser beam over the specimen area. However, in many assays, including all of those that use blood as the specimen, the specimen is heterogeneous, with the cells of

normal interest for the assay being only a small fraction of the total specimen's cells. For example there are normally 1000 times as many red blood cells as each specific type of white blood cell. The presence of irrelevant cells therefore results in increased processing time to analyze a given number of cells of interest, if irrelevant cells must also be measured and analyzed, since a larger area is required to place all the cells on a surface without overlap of the cells. An expedient of simply placing the entire sample in a desirably small scan area will however result in multiple layers of irrelevant cells being present, with overlaying of the cells of interest, thereby actually interfering with the measurement process itself. It is thus highly advantageous if only cells of interest remain in the small area to be scanned and irrelevant cells are removed prior to measurement.

In prior art methods, specifically selected analytes are made to adhere to a surface by capture of the analyte on a surface by means of a reagent which binds thereto, and thereafter the quantity of the analyte itself is measured (see U.S. Patent No. 5,637,469). However, often the applicable reagent required to cause cell adherence will not always result in the proper subset (i.e., cells of interest) adhering to a surface without irrelevant cells.

SUMMARY OF THE INVENTION

It is therefore an object of the present invention to provide a means for increasing the efficiency of laser scanning cytometry by limiting the scanning area of a heterogeneous cell specimen, with removal of irrelevant cells and the like from a designated limited area, and rendering cells of interest in the limited area statistically sufficient to provide meaningful measurements.

It is another object of the present invention to separate from a heterogeneous specimen, a set of cells containing the cells of interest, by causing the set of cells to be located in the limited area, thereafter discriminating between a subset of

cells of interest and other cells of the set, not of interest, and then performing measurements only on the subset of cells of interest. Hereinafter, reference to "cells of interest" includes a "cell set (e.g., red blood cells, white blood cells, microorganisms, etc.) containing a subset of cells of interest" (e.g., monocytes) unless otherwise indicated.

It is another object of the present invention to identify a subset of cells of interest from among cells adhering to a limited area of a surface by a marker reagent which can be measured by light sensors of a laser scanning cytometer.

It is yet another object of the present invention to use one or more additional marker reagents that will identify characteristics of cells of interest which will be measured by additional light sensors in the laser scanning cytometer.

It is a further object of the present invention to thereby enable laser scanning cytometers to be used as a point-of-care instrument to be used by unskilled operators, wherein a specimen is processed in the laser scanning cytometer within a disposable cartridge, without user intervention, and wherein the cartridge contains all necessary reagents needed to react the specimen with fluorescent dyes, as well as providing means for removing irrelevant cells and placing relevant cells in a small area on a surface that will be scanned by the cytometer.

Generally the present invention comprises a method and device for adhering cells of interest (or a set of cells containing cells of interest as a subset) to a limited area of a stationary specimen substrate such as a microscope slide, for examination by a laser scanning cytometer. The method of the present invention comprises the steps of:

a) treating a heterogeneous cell specimen sample with adhering means which bind to cells of interest or to a selected set of cells including the cells of interest as a subset thereof;

b) adhering cells bound with the adhering means to a limited area substrate by secondary adhering means;

c) if relevant, discriminating with discrimination means between adhered cells of the subset of interest from other cells of a set if a selected set of cells is adhered; and

d) analyzing cells of interest with a laser scanning cytometer in the limited area of the substrate.

Alternatively, steps "a" and "b" in the above method, can be replaced with the step of the surface itself being initially provided with the adhering means, whereby the cells of interest, or set of cells containing the cells of interest as a subset, are adhered to the surface in situ, without pre-treatment of the specimen sample. Additionally, irrelevant cells can be separated out with the use of a porous membrane.

In a particularly preferred embodiment attuned to actual usage, the analysis and separation is conducted in three separate steps:

a) there is an initial separation of a general class of cells from the overall heterogenous sample for adhesion to a surface by means of a relevant reagent;

b) cells of interest which are adhered among other related but irrelevant cells (i.e., the actual cells for which measurements are exclusively contemplated) are marked with a marker reagent (different from the reagent used to adhere the cells) for the identification thereof, separate from the other adhered cells; and

c) using one or more reagents for the determination of specific properties (not merely a count) of the marked cells of interest.

As a result of this procedure, in accordance with the present invention, clinically important complex properties of cells, such as their activation status or binding to other cells can be determined.

In a specific embodiment of the device of the present invention, used in effecting the above method, a slide is provided with a fluid specimen loading conduit, wherein non-impeding gating means, having the secondary adhering means, is

provided in a minimal area of the conduit. Said gating means effects adhering of the cells having the adhering means attached thereto with application of the secondary adhering means in a pre-selected limited gating area.

In accordance with the method of the present invention, there are three different techniques for adhering specific cells of a type to limited area surfaces of a specimen substrate.

In the first technique, cells of a heterogeneous specimen are reacted with paramagnetic particles that have been coated with material that will bind the particles to an antibody and the antibody is selected so that it will bind to an antigen on the surface of the cell type of interest in the specimen. The heterogeneous specimen is then reacted with the coated paramagnetic particles whereby the particles bind to the cells of interest (or cells of a set including a subset of cells of interest). Techniques for manufacturing and using such particles in biologic assays are described in U.S. Patents Nos. 3,933,997; 3,970,518; 4,018,886; 4,047,814; 4,219,411; 4,230,685; 4, 695, 393; 5,411,863; and 5,543,289.

The cells of interest are adhered to the substrate and thereby separated from irrelevant cells by any of the following procedures:

a) the suspension can be made to flow over a magnetic field in a flow chamber to retain cells of interest on the surface. In all of the above, the magnetic material comprises the secondary adhering means (this is similar to procedures described in U.S. Patents Nos. 4,219,411; 4,731,337 and 5,053,344).

b) the cells of interest can be separated from the irrelevant cells by moving a magnetic field through the suspended cells within the stationary fluid, thereby causing the cells to follow the magnetic field to an area within or outside the original stationary suspension (see U.S. Patents Nos. 3,985,649, 3,712,472, 4,272,510, 4,292,920, and 5,567,326). Prior art also describes moving particles and cell from one

liquid phase to a second liquid phase by a magnetic field (e.g., U.S. Patents Nos. 4,777,145 and 5,279,936).

The second method of adhering selected cells to a surface area is to directly deposit antibodies on the substrate surface in the desired limited area. The specimen is made to come into contact with the surface, whereupon antigens on the surface of cells of interest bind to corresponding antibodies on the surface, and irrelevant cells are washed away. Reagents such as cellulose binding domain are commercially available for enhancing antibody to surface binding to achieve antigen capture. This technique has been reviewed by Norden et al, *An Experimental Model of Affinity Cell Separation*, Cytometry **16**:15-33 (1994).

A third method of adhering selected cells to a surface area in accordance with the present invention is to use a membrane filter with pores that will selectively allow specific types of cells to pass through the membrane while retaining others. This methodology has been commonly employed for separating blood cells and advantageously for separating leukocytes from red blood cells and platelets, which readily pass through a 5 micron sized pore while retaining leukocytes on the membrane surface. Such filters are commercially available from numerous suppliers such as Osmotics, Livermore, CA. These membranes may be layered over an absorbent material such as MFS Borosilicate microfiber manufactured by Micro Filtration Systems, Dublin, CA, with the specimen being made to flow over the membrane surface, thereby capturing selected cells, while passing irrelevant cells and fluid through the pores. Capillary action may be utilized to cause flow of specimen, after it has been mixed with marker reagents, to the filter surface. The absorbent material may be placed below the membrane in a chamber that serves as the waste reservoir.

Though the prior art is replete with magnetic, antibody adhesion, and filtering techniques, the prior art has not taught or suggested the use of magnetic, affinity adherence or

filtering for cell constituent analysis such as with laser scanning cytometry.

Commonly, with flow cytometry, cells are reacted with fluorescent dye conjugated antibodies and then washed. Multiple centrifugations are employed to separate cells from reacting reagents to perform the reaction and washing steps. Also, physical separation techniques such as Ficoll-Hypaque or cell analysis techniques are employed to reduce the numbers of irrelevant cells. In accordance with the present invention, laser scanning cytometry is utilized in order to exploit the utility of solid phase assay as described.

Other objects, features and advantages of the present invention will become more evident from the following description and drawings in which:

SUMMARY OF THE DRAWINGS

FIGS. 1a and 1b are schematic representations of disposable specimen loading devices constructed in accordance with a preferred embodiment of this disclosure.

FIG. 2 is a schematic representation of the optical measurement system used in effecting measurements of the specimen on the devices shown in Figures 1a and 1b.

FIG. 3 is a rendering of photo sensor data from a laser scanning cytometer, as the optical measurement system of FIG. 2.

FIG. 4 is a schematic depiction of a disposable slide device as used in Figures 1a and 1b, with loading conduit and gated area shown.

FIGS. 5a and 5b are data from a laser scanning cytometer of an example of an assay employing an embodiment of the disclosure as set forth in a scattergram and histogram respectively.

FIGS. 6a-c are cell scattergrams of various parameters prepared from data from a laser scanning cytometer of an example of an assay of a specimen sample.

FIG. 6d is a histogram obtained from the data of FIG. 6a.

FIGS. 7 are two histograms obtained from laser scanned cytometer data from platelet bound CD42b-PE-separated and superimposed to obtain a third histogram.

FIGS. 8, 9a and 9b are additional cell scattergrams obtained from data obtained from specimens assayed by a laser scanning cytometer.

FIGS. 10a-b are histograms of superimposed histograms after assay of two specimens.

FIG. 11 is a rendering of an analysis instrument used in the specimen preparation and analysis in accordance with the present invention.

FIG. 12a is a dot plot of the position of each granulocyte of a sample on the filter of another embodiment of the present invention.

FIG. 12b is a histogram of the laser scanning cytometer CD11b-FITC fluorescence per cell of the sample shown in FIG 12a and a second sample.

FIGS. 13a and 13b are two sets of superimposed histograms of the orange fluorescence values per cell of other specimens.

DETAILED DESCRIPTION OF THE INVENTION

The method and device of the present invention, when combined with use of laser scanning cytometry technology allows the development of an instrumental system that can perform a number of specific cell constituent assays, whereby an unskilled user can obtain rapid results as a point-of-care instrument. A number of clinically important assays are described below, by way of Examples showing use of the present invention, which have been developed using flow cytometry but have not been employed in routine clinical medicine, because of problems inherent with the use of flow cytometry.

EXAMPLE I

Among assays performable by laser scanning cytometers are tests for activation of specific blood cells caused by sepsis in newborns or adults, which can be detected by measuring the activation of blood granulocytes resulting from interaction of

these cells with bacteria. (See e.g., Davis et al *Neutraphil CD64 expression: Potential diagnostic indicator of accute inflamation and therapeutic monitor of interferon - γ therapy*, Laboratory Hematology 1:3-12 (1995). Leukocytes from whole blood are captured on a small area surface of a specimen substrate by a CD45 antibody, a fluorescent dye is conjugated to a CD15 to bind to granulocytes and thereby used to identify granulocytes. The fluorescence of a second dye conjugated to the antibody CD11b which is expressed on the granulocyte surface as a function of the cell's activation state is measured by a laser scanning cytometer. A test for adult sepsis is made by substituting the antibody CD64 for CD11b.

Additionally, in adults, CD11b granulocyte activation is used to determine the condition of patients following angioplasty or stent surgery. The activation of another constituent of blood, platelets as measured by antibodies such as AAC-2 to p-selectin, after their capture to a surface by an anti-platelet antibody or thrombin, and their identification by antibodies such as CD42b is used in an assay for cardiovascular disease.

EXAMPLE II

A sensitive assay for early detection of myocardial infarctions (AMI) as well as other vascular pathologies has been devised and tested using flow cytometry (described in U.S. Patent No. 5,503,982). This assay relies on the discovery that after arterial damage, circulating platelets become activated resulting in p-selectin's transport to the platelet surface where it binds to blood monocytes. A high ratio of monocytes that are complexed with platelets to uncomplexed monocytes in a patient blood specimen is an indicator of an AMI.

In accordance with the present invention, leukocytes from whole blood are captured on a small area surface by a CD45 antibody and a fluorescent dye is conjugated to a CD14 antibody which binds to monocytes, and can be used to identify them. The fluorescence of a second fluorescence dye conjugated to a CD42b

or CD61 antibody, which binds to platelets, is measured. The ratio of the number of monocytes with second fluorescence to the total number of monocytes is used in the assay determination.

EXAMPLE III

The method of the present invention is used to assay for drug occupancy on a cell surface. One important clinically useful assay of this type is to measure whether platelet protein GPIIb/IIIa is bound to a drug or is capable of binding to fibrinogen (described in U.S. Patent No. 5,440,020). Such drugs are being developed for treatment of various cardiovascular diseases. It is necessary to monitor the level of these drug's occupancy, since over-treating will lead to other conditions such as stroke. Techniques for performing diagnostic assays of these drugs are disclosed in U.S. Patent No. 5,114,842.

In accordance with the present invention, blood is activated by an ADP agonist to cause fibrinogen binding to non drug occupied platelets. Thereafter, platelets from the whole blood specimen are captured on a small area substrate surface by a CD42b antibody. A fluorescent dye is conjugated to a CD41 antibody which binds to the GPIIb/IIIa protein in a region separated from the fibrinogen binding region (U.S. Patent No. 5,372,933). The fluorescence of a second fluorescent dye conjugated to a RIBS antibody which binds to fibrinogen when it, in turn, is bound to platelet GPIIb/IIIa, is measured. The ratio of the second fluorescence to first fluorescence intensity for each captured platelet is measured and the distribution of this ratio and computed derivatives of it are reported as the assay determination.

The method of the present invention may also be used for immunophenotyping, for example, counting T4 cells for AIDS patient monitoring or for diagnosing various leukemias. This is presently the major application for centralized flow cytometry instruments and is clinically employed because results are not required immediately so that an expensive, labor intensive flow cytometer can be employed.

The present invention, as used with a laser scanning cytometer, is however separately useful because of the very high volume clinical assay of white blood differential counts since laser scanning allows for the relocation of events for visual observation, which is not possible with flow cytometry. With use of the present invention, leukocytes from whole blood are captured on a small area surface by an antibody such as CD45, a fluorescent dye is conjugated to an antibody such as CD3 that will bind to T lymphocytes and can be used to identify them, and the fluorescence of a second fluorescence dye conjugated to an antibody such as CD4 which binds to T4 helper lymphocytes, is measured. The ratio of the number of CD4 positive cells with second fluorescence to the total number of T lymphocytes is used as the assay determination. This can be extended to many other CD antibodies to detect other cell types. It is contemplated that the methods of the disclosure can be extended to more than detection of one type of cell by using more than two fluorescent dyes conjugated to antibodies.

DETAILED DESCRIPTION OF THE DRAWINGS AND THE PREFERRED EMBODIMENTS

With reference to Figs. 1a and 1b, a specimen such as blood from a patient is placed in a closed end tube 1. Tube 1 may be a standard vacutainer used to draw blood, with a rubber sealing insert 12 at its top. Tube 1 is inserted into sleeve 13, of the disposable 2, thereby causing tubes 3 and 4 to pierce insert 12. The disposable 2 (preferably made of molded plastic material) contains three chambers 5, 7, and 8 and two ports 6 and 9. Reagents are contained within chamber 5 of the disposable. In one embodiment, in which magnetic particles coated with an antibody are used to cause cells to adhere to a surface, chamber 5 contains coated magnetic particles. In a preferred embodiment, chamber 5 contains two or more antibodies to cellular antigens, each of which is conjugated to a different fluorescent dye. Chamber 5 is connected to tube 4 as well as port 6. The assembly consisting of the specimen tube 1 and disposable 2 is inserted

into the specimen entry port 81 of the analysis instrument 80 shown in Fig 11.

The assay is activated by the user by pressing the enter key of the instrument beginning the test cycle. Port 6 which is connected to a pressure source in the instrument is pressurized then cycled in pressure, to cause the reagent contained in chamber 5 to enter through tube 4 and mix with the specimen in tube 1. The reagent contains two or more antibodies, each conjugated to a different fluorescent dye. In preferred embodiments these are fluorescein isothiocyanate (FITC) and phycoerythrin (PE) which are excited by the wavelength energy of an Argon ion laser. Other dyes such as CY3, CY5, APC, CY5.5. or CY7 could be used for excitation by other gas or solid state lasers. The reagent of the preferred embodiment in which cells are adhered by a magnetic field, also contains magnetic particles coated with an antibody such as CD45 which will bind to cell surface antigens such as those of white blood cells for CD45. Such particles are commercially available from Perceptive BioSystems, Framingham, MA. In another embodiment in which cells are directly captured by an antibody coated on a surface, the magnetic particles are not part of the reagent.

After an incubation period, during which time the specimen is mixed by varying the pressure through port 6, the pressure through port 6 is maintained while port 9 is opened. The specimen mixture flows through tube 3 to chamber 7. Chamber 7 has dimensions of 50 to 400 microns in depth so that the specimen cells will flow close to the lower surface where there is a magnetic field gradient. The chamber 7 width is set at a value depending on the number of cells necessary to be captured for good statistical results, as cells will be captured perpendicular to the flow. A typical chamber width is about 5 mm. A magnet is placed in the instrument so as to produce a strong magnetic gradient along a line perpendicular to the flow at the area 10. This is accomplished in the Examples by using a rectangular bar magnet with the pole face directed against the

chamber 7, and the magnet edge placed at the centerline of area 10. To enhance the magnetic gradient, the chamber 7, can contain a paramagnetic wire with a triangular cross section embedded in the disposable perpendicular to the flow, so that the wire apex is near the cell flow and the base is in contact with a magnet. As the specimen flows through chamber 7, cells that have bound to the magnetic particles will adhere in the area 10 (i.e. which is, in effect, a pre-determined "gating area" for the cells). The port 6 is then connected to a source of phosphate buffered saline which is made to flow through chamber 5, and into chamber 7, thereby washing irrelevant cells from surface area 10. Waste is collected in chamber 8. Cells are collected over the area 10 (a non-flow impeding gate) defined by the width of chamber 7 and the distribution width characteristics of the magnetic field gradient. The field and chamber dimensions are appropriately adjusted so that the cells of interest are captured as a monolayer for the specimen used. In other embodiments, cells can be captured using an antibody coated surface area 10, in place of a magnetic field and magnetic particles.

After completion of the cell capture phase, surface area 10, is scanned by a laser scanning cytometer as schematically shown in FIG. 2. The scanning may be preferably done in the same instrument used to capture the cells, or can be done within a second independent instrument. The disposable 26, is held to movable stage 33. The instrument contains a laser 20, such as an Argon ion, HeNe, or solid state laser and the choice will depend on the fluorescent dyes used. The laser beam 21 is reflected by dichroic mirror 22, and computer controlled scanning mirror 23. Such scanning mirrors are commercially available, for example, from General Scanning, Watertown, MA. The beam is passed through lenses 24 and 25, to produce a line scan focused at area 10. The dimensions of this line scan are preferably the width of the cell capture area in scan extent and 5 to 10 microns in beam diameter. The disposable 26 is moved perpendicular to the scan by the stage 33, to raster scan all of area 10. As each cell

encounters the laser beam it emits fluorescent light proportional to the amount of each fluorochrome in the scan spot. This fluorescence is collected by lens 25 and imaged back through the laser light path 21, where it is collimated as it passes through dichroic mirror 22. In a preferred embodiment, two fluorescence emissions are measured. Dichroic mirror 27 splits the emission into two wavelength ranges, optical filters 28 and 31 further define the wavelength ranges detected by photo sensors 29 and 32. In an embodiment using an antibody coated area 10, it is contemplated that a third sensor measuring forward angle scatter will be used to find cell data and isolate data belonging to individual cells. This sensor will consist of a light blocking bar and photo sensor placed along the laser light path below disposable 26.

The data signals from all photo sensors are simultaneously digitized at a fixed rate, such as at 625,000 Hz. As the scan beam passes over area 10 due to the motion of the scan mirror and disposable platform, a raster of digital data or pixel values is generated and stored in memory of a computer. FIG. 3 is a representation of data from one sensor as fluorescent emitting cells are scanned where the density at each pixel position is representative of the data value. The data from a typical cell is represented by the pixels 30. Cells are found if a set number of contiguous values above a set threshold are found for any sensor's data. Alternatively a scatter sensor may be used for detecting the presence of and isolating cell data. The computer program first isolates the data belonging to each cell found. It generates a contour 31' surrounding the cell at the threshold value. The largest contour of the sensor contours is selected and this is enlarged so that all cell data is used. The pixel values within each contour are then summed. Two additional contours 32' and 33 are constructed by the computer a set distance from contour 31'. For each sensor, the pixel values between these contours are averaged to determine a background level which is subtracted from the corresponding cell sensor

sums. The integrated value result is proportional to the fluorescence emission from that cell as detected by each sensor and in turn is proportional to the number of a specific antigen or other constituent bound to fluorescent dye molecules on the cell.

Additional information, such as the contour area equal to the number of pixels in the contour of each cell or the maximum pixel value in the contour can be computed and may be used to distinguish single cells from cell clusters and distinguish cell states.

The integrated values determined as above are used to determine the contents of information displayed to the user. This data may be displayed as two parameter scattergrams, histograms, or numeric values as described in the examples. The result can be displayed on a screen, alphanumerical display, or printed form.

The following examples are provided as evidencing test results from laser scanning cytometry of various cell determinations and characterizations in accordance with the present invention.

EXAMPLE 1

This example describes an experiment to measure the cell capture efficiency for a specific disposable design using magnetic particle capture to adhere cells to a surface in a defined area. Whole blood was diluted 1:1 with phosphate buffered saline (PBS) containing an antibody conjugated to fluorescein isothiocyanate (FITC). For this example the mouse anti-human antibody CD45 which binds to all leukocytes was used. After an incubation period the cells were washed with PBS and 50 microliters of Perceptive Biosystems magnetic particles conjugated to an anti-mouse antibody were added to 100 microliters of the specimen.

A disposable device 40 shown diagrammatically in Fig. 4 was constructed by adhering a 200μ thick adhesive coated mylar template 41 with a 5 mm wide channel to a standard microscope

slide. A standard glass cover slip 43 was adhered to the center of the slide so as to form a 200μ x 5 mm flow channel **42**. A bar magnet was glued below the slide so that one pole was in direct contact with the back surface of the slide and perpendicular to the flow path. In this way two strong magnetic gradients are placed along the specimen flow path and one would expect that maximum capture of magnetically coated cells would occur in proximity to the two edges of the magnet.

The specimen was pipetted into the right side of the flow channel and flowed by capillary action through the channel. A cotton wick was placed at the left end of the flow channel and PBS was then pipetted into the flow channel to wash away all irrelevant cells not adhering to the slide. This resulted in the appearance of a line 44 from the excess magnetic particles adhering to the slide surface at a position coinciding with the first edge of the magnet where the magnetic field gradient is strongest.

The slide disposable was placed on the microscope stage of a CompuCyte LSC®, CompuCyte Corporation, Cambridge, MA., laser scanning cytometer and a portion of the cover slip area was scanned to locate the position of each cell found based on detection of FITC fluorescence. The location of each cell can be displayed as a scattergram 50 of Fig. 5a, in which each dot represents a cell at the X and Y coordinates of the axes. The number of cells as a function of position along the flow path can be plotted as a histogram 51 of Fig. 5b, in which numbers of cells at each X position is plotted.

If it is assumed that the magnetic gradient at the leading and trailing edges of the magnet are equal, it is possible to calculate the efficiency of setting and holding cells that can be captured in place during the specimen and wash fluid flows through the chamber. In this experiment the number of cells captured near the first gradient is approximately twice the number near the second gradient. With the small number of cells captured between the gradients, the efficiency is calculated as

approximately 50%. It should be noted that provided there is no biased capture of a subset of the cells of interest, practice of this invention does not require high efficiencies. It requires capturing statistically significant numbers of cells within a small area that can be scanned.

EXAMPLE 2

A disposable configured as described in Example 1 was used in all of the remaining examples. In this example, cells are captured with the same antibody used to detect their presence with a first fluorochrome, and a property of the cells is measured using a second antibody and fluorochrome.

A specimen of whole blood was mixed 1:1 with PBS containing a FITC conjugated mouse anti-human antibody CD42b which binds to platelets and a phycoerythrin (PE) conjugated mouse anti-human antibody ACC-2 which binds to p-selectin antigen found on the surface of activated platelets. PE fluoresces at a wavelength that can be separated from FITC fluorescence by appropriate optical filters. This example is intended to show the number and extent of activated platelets in a patient blood specimen. After incubation, 50 microliters of anti-mouse antibody conjugated magnetic particles were added to 100 microliters of the mixture, incubated and added to the flow chamber followed by a PBS wash. This was repeated with a second control blood specimen without the ACC-2 antibody. The resulting LSC displays after assaying these two specimens is shown in Figs. 6a-d. The position distributions of the detected platelets in the control specimen are shown as a scattergram 60 of Fig. 6a and a histogram 61 of Fig. 6d and are similar to Example 1. The spectral overlap compensated scattergram 63 of Fig. 6c shows the level of activation based on ACC-2 antigen measurement plotted versus CD42b antigen per platelet. The data was compensated for filter spectral overlap as practiced in flow cytometry phenotyping. The number of events in each of the four quadrants of display 62 of Fig. 6b were counted and 83% of the platelet events had a relatively high level of ACC-2 p- selectin expression as

compared to the control specimen's scattergram display 63 of Fig. 6c in which the count in this quadrant was 12%.

It is understood that other antibodies can be used to measure cell activation such as antibodies that will bind to GPIIb/IIIa or fibrinogen, as well as p-selectin of platelets. It is also contemplated that this method will be used to measure granulocyte activation using either CD11b or CD64, or other antibodies expressed on activated granulocytes. It is also understood that the first antibody can be different than the antibody used to capture the cells of interest.

EXAMPLE 3

This is an example of application of the preferred embodiment in which cells are captured to a small surface area with a first antibody, a subset of cells is identified with a second antibody, and a characteristic of the cells of the subset is measured with a third antibody. This example demonstrates an assay to determine the percentage of monocytes complexed with platelets. It has been shown in the prior art that cardiovascular injury results in the activation in circulating blood, of platelets which then in turn bind to monocytes. An assay of the ratio of platelet bound to non-platelet bound monoctes is an indicator of cardiovascular injury such as an acute myocardial infarction.

Two samples of whole blood from the same individual were obtained. Two micromoler ADP and 5 millimolar GPRP were added to one sample to activate the platelets. Platelets, when activated, bind to some leukocytes including monocytes. Each sample was mixed in a 1:1 ratio with PBS containing three antibodies, the first mouse anti-human CD45 conjugated to Perceptive Biosystems magnetic particles. CD45 binds to a common leukocyte antigen on white blood cells. The second antibody, mouse anti-human CD14 was conjugated to FITC and binds to monocytes. The third antibody, mouse anti-human CD42b, which binds to platelets, was conjugated to the dye phycoerythrin (PE). The samples were then fixed by adding paraformaldehyde to a 1% concentration to

further inhibit platelet activation. Each mixture was placed in a disposable as described in Example 1, followed by PBS to wash irrelevant cells from the surface. Each slide was assayed on the LSC laser scanning cytometer, measuring fluorescence from each of the dyes. Cells were detected and data from each cell was contoured based on green fluorescence from the FITC bound to CD14 on monocytes. Fig. 7 shows two superimposed histograms of the maximum value within each contour of orange fluorescence from the platelet bound CD42b-PE. The non-activated sample curve 71 is clearly differentiated from the assay result 72 of the activated sample indicating that the preferred embodiment can be used to assay for platelet monocyte complexes in a blood specimen.

This experiment was repeated using the same dye conjugated antibodies and capture reagent, however fresh undiluted and unfixed blood was used for both the unstimulated and stimulated specimens. Ten micromolar ADP was used to stimulate adhesion of platelets to monocytes in this experiment. The results are shown in Fig. 7 in which curve 73 represents the data from the unstimulated specimen and 74 represents the data from the ADP stimulated specimen, measuring the total fluorescence of CD42b-PE of each found cell. In the separate graph of Fig. 7, 75 represents the data from the unstimulated specimen and 76 represents the data from the ADP stimulated specimen, measuring the maximum fluorescence of CD42b-PE of each found cell. The two populations from the stimulated and unstimulated specimens of the same fresh blood are clearly distinguishable. It is noted that other platelet antibodies such as CD61 and CD 41 may be preferable to CD42b for the assay of this example because the antigen to which CD42b binds may be decreased when platelets are activated.

EXAMPLE 4

Using the method of the preferred embodiment as in Example 3, the percentage of lymphocyes expressing the T4 antigen was

assayed. This is a common assay performed on flow cytometers for monitoring AIDS patients. In this example, the specimen was mixed with CD45 magnetic particles to capture leukocytes as above, The reagent mixture, in this example, contained mouse anti-human CD3 antibody conjugated to PE and mouse anti-human antibody CD4 conjugated to FITC. CD3 binds to antigens found on T lymphocytes while CD4 binds to T4 or helper T lymphocytes.

The slide was assayed as described in Example 4 resulting in the scattergram data of Fig.8. CD4 expressing lymphocytes have higher green fluorescence than the remaining T lymphocytes and appear in the upper cluster of the scattergram of CD4 versus CD3. Using gating regions this assay showed that approximately 58% of T cells were T4.

EXAMPLE 5

This example uses the preferred embodiment to demonstrate the common white blood cell differential count. A whole blood specimen was prepared as in examples 3 and 4, using CD45 conjugated to magnetic particles and the monocyte specific mouse anti-human antibody CD14 and the granulocyte specific mouse anti-human antibody CD15. CD14 was conjugated to FITC and CD15 was conjugated to PE. The slide was assayed twice, the first time using green fluorescence data to determine the contour and the second time using orange fluorescence to determine the contour. This was done this way because the CompuCyte LSC does not have the means to simultaneously use two sensors to segment cell data. The resulting data is shown in Figs. 9a and 9b in which the two scattergrams represent data from each of the two assays respectively. The orange fluorescence gated scattergram shows 95% of the cells expressing orange fluorescence are CD15 positive and are granulocytes. Approximately 2920 cells were counted in quadrant region 4. The green fluorescence gated scattergram shows 92% of the cells in quadrant region 1 expressing high levels of CD14 and are monocytes. Approximately 800 cells were counted in region 1.

EXAMPLE 6

This example uses the preferred embodiment to demonstrate the determination of activation of whole blood granulocytes by the agonist f-Met-Leu-Phe (fMLP). Two samples of the same blood specimen were obtained. To one sample at 37° C, 10 μM fMLP was added and that sample was incubated for 10 minutes to cause activation of the sample's granulocytes. Both samples were mixed 1:1 with PBS containing two antibodies. Mouse anti-human CD15 was conjugated to FITC and binds to granulocytes. The second antibody, mouse anti-human CD11b which binds to an activation antigen of granulocytes was conjugated to the dye phycoerythrin (PE). After an incubation period, paraformaldehyde was added to a final concentration of 1%. Fifty microliters of anti-mouse antibody conjugated magnetic particles was then added to 100 microliters of each mixture, incubated and added to the flow chamber followed by a PBS wash. Each specimen was assayed on the LSC cytometer, detecting and contouring events based on green fluorescence from the FITC bound to the granulocytes. The orange PE fluorescence resulting from the binding of CD11b to the granulocyte activation antigen was measured. The resulting superimposed LSC histogram displays after assaying these two specimens is shown in Fig. 10a. The distribution of fluorescence per cell of the unactivated sample had the result shown as 91 and the activated sample had the distribution result 92. These distributions are clearly distinguishable from each other.

This experiment was repeated with undiluted blood using the same dye conjugated antibodies and 10 μM fMLP stimulant. However, in this experiment the specimens were not washed and the specimens were incubated for 30 minutes with magnetic particles conjugated to CD45 antibody to capture them on to a surface. The results of assaying these specimens on a CompuCyte Corporation LSC cytometer are shown in Fig. 10b, in which blood assayed within 2 hours of draw is represented as curve 93, blood assayed 6 hours later is represented as curve 94, and a stimulated specimen of the same patient's blood is represented

as curve 95 in which the distribution of per cell CD11b-PE fluorescence is shown. The stimulated specimen assay data is clearly distinguishable from both non stimulated specimens.

It should be understood that other means of capturing cells not using antibodies such as coating magnetic particles or surfaces with lectins are contemplated. Also means of distinguishing cells or subsets of cells using dyes not conjugated to antibodies are contemplated. For example, dyes such as propidium iodide taken up by cellular nucleic acids are contemplated as are chemical substrates producing fluorescent end products as a result of interaction with cellular constituents. The use of nucleic acid probes as a marker is also contemplated.

For example, bacteria contained in blood, pharmaceuticals, or food may be captured onto a surface using lectins coated to the surface or conjugated to magnetic particles (See e.g. Payne et al *The use of iImmobolized lectins in the separation of Staphylococcus aureus, Escherichia coli, Listeria and Salmonella spp. From pure cultures and foods, Journal of Applied Bacteriology* 73:41-52 (1992). Bacteria can be counted by using nucleic acid specific dyes taken up by all bacteria. (See the review, Kepner et al, *Use of Fluorochromes for Direct Enumeration of Total Bacteria in Environmental Samples: Past and Present,* Microbiological Reviews, 58:603-615 (1994). The surface can be scanned and in this case fluorescent events counted. Additionally, a second dye (available from Molecular Probes, Eugene, OR.) can be used to determine if the bacteria are alive or dead and can be added to differentially count live bacteria. Third, bacterial specific antibodies can be used to determine the type of bacteria on the surface (See e.g. Vesey et al, *Evaluation of Fluorochromes and Excitation Sources for Immunofluorescence in Water Samples,* Cytometry 29:147-154 (1997). Fourth, ribosomal RNA specific nucleic acid probes can be used to identify bacteria. This is reviewed by Amann et al, *Phylogenetic Identification and In Situ Detection of Individual*

Microbial Cells without Cultivation, Microbiological Reviews 59:143-169 (1995).

It is also contemplated that the surface used to capture the bacteria may be embedded in a growth medium, allowing the live bacteria to remain viable. In this case, after a period of time to allow the bacteria to reproduce, the capture area may be scanned a second time and the specimen reassayed. Since laser scanning cytometry records the position of each cell, as described in the examples, it is possible to determine changes in fluorescence of any event found. Event contouring can be adjusted to encompass a large enough area so proximate bacteria are within the same contour. If nucleic acid per bacteria, for example, is measured, viable bacteria will have increased amounts of DNA per event measured during the second assay, and their viability indicated. The patterns of located events (as shown and described in EXAMPLE 1) for the first and second assays can be compared by software to determine the correspondence of each events data in the first and second assays. The corresponding fluorescence values will then indicate bacterial growth by virtue of increases in total fluorescence or size properties between the two assays.

It is also contemplated that, with the antibody coating cell capture method, more than one capture area will be used, each coated with a different antibody, so that different types of cells will be captured in different areas of the surface. This will allow for more complex assays of multiple cell types without the need to employ multiple fluorescent dyes and multiple instrument sensors to detect these fluorescences. Additionally one specimen may be tested for multiple assays.

The following examples are indicative of the manner in which the third embodiment of the cell separation is effected in accordance with the present invention. In such embodiment the selective cell separation is effected by means of a filtration system as described.

EXAMPLE 7

This example exemplifies the embodiment in which all leukocytes from a whole blood specimen are separated from red blood cells by a membrane with 5 micron pores. The example demonstrates an assay for determination of the activation of whole blood granulocytes by the agonist f-Met-Leu-Phe (fMLP).

Two samples of the same blood specimen were obtained. 10M fMLP was added to one sample at 37°C, and the sample was incubated for 10 minutes to cause activation of the sample's granulocytes. Each sample was then separately mixed in a 1:1 ratio with PBS containing two antibodies. A first antibody, mouse anti-human CD15 that binds to granulocytes was conjugated to the dye phycoerythrin (PE). The second antibody, mouse anti-human CD11b that binds to an activation antigen of granulocytes was conjugated to the dye fluorescein isothiocyanate (FITC).

After an incubation period, 100 microliters of each mixture, was pipetted into the center of an Osmotics (Catalog no. 10572), 5 micron pore size, 13 mm membrane filter placed over MFS Borosilicate microfiber absorbent material (Micro Filtration Systems, Dublin, CA). This was immediately followed by the pipetting of 200 microliters of PBS onto the membrane center surface. Each filter was removed, placed on a microscope slide and covered with a slip and assayed on the laser scanning cytometer. Granulocytes were detected and contoured based on orange fluorescence from the PE bound to the granulocytes. The green FITC fluorescence resulting from the binding of CD11b to the granulocyte activation antigen was measured for every contoured event. Since the LSC cytometer is capable of recording and displaying the position of each found event, it was used for such purpose. The positions of each granulocyte on the filter, for the first specimen, is displayed as a dot plot 101 of Fig. 12a. The resulting superimposed LSC histogram displays of CD11b-FITC fluorescence per cell, after assaying these two specimens are shown as Fig. 12b. The distribution of fluorescence per cell of the unactivated sample is shown as 103 and the activated sample is shown as distribution 104. These distributions are

clearly distinguishable from each other. The histogram display was divided into two regions by the boundary line 105 and the counts of cells on the right (positive CD 11b) side of the boundary was determined for each of the unstimulated and stimulated specimen. For these assays the unstimulated count was 87 of 15,882 or 5.1% of the total cells counted and the stimulated specimen's count was 7342 of 9043 or 81.2%.

EXAMPLE 8

This is an example of an application of the third embodiment in which cells are captured on a membrane of small surface area, wherein a subset of cells is identified with a first antibody, and wherein a characteristic of the cells of the subset is measured with a second antibody. The example illustrates an assay of determination of the percentage of monocytes complexed with platelets.

It has been shown in the prior art that cardiovascular injury results in the activation, in circulating blood, of platelets which in turn bind to monocytes. An assay of the ratio of platelet bound to non-platelet bound monocytes is an indicator of cardiovascular injury, such as an acute myocardial infarction.

Four samples of whole blood from the same individual were obtained. Two micromolar ADP and 5 millimolar GPRP were added to two samples to activate the platelets. Platelets, when activated, bind to some leukocytes including monocytes. Each of the four samples was separately mixed in a 1:1 ratio with PBS containing two antibodies. The first antibody, mouse anti-human CD14 was conjugated to FITC, which binds to monocytes, was used in all specimens The second antibody, mouse anti-human CD42b, was added to one activated and one non-activated sample, and the antibody, mouse anti-human CD62, was added to one activated and one non-activated sample. Both antibodies, which bind to platelets, were conjugated to the dye phycoerythrin (PE). 100 microliters of each of the mixtures were pipetted to the center of a filter as described in Example 7, followed by PBS to wash

irrelevant cells from the surface. The membranes were transferred to a slide and a slip was placed over each membrane. Each slide was assayed on the LSC laser scanning cytometer, measuring fluorescence from each of the dyes. Cells were detected and data from each cell was contoured based on green fluorescence from the FITC bound to CD14 on monocytes. Figs. 13a and 13b show two sets of superimposed histograms of the orange fluorescence values per cell, the histograms 106 from the platelet bound CD42b-PE specimen, and the histograms 109 from the platelet bound CD62-PE specimen. The non-activated sample curve 1077 is clearly differentiated from the assay result 108 of the activated sample as is the non-activated curve 110, differentiated from activated curve 111. The histogram displays were divided into two regions by the boundary lines 109a and 112 respectively and the counts of cells on the right side of the boundary consisting of complexed monocytes and platelets (positive CD42b or CD62) was determined for each of the unstimulated and stimulated specimens. For these assays the unstimulated count using CD42b was 111 of 2408 or 4.6% of the total cells counted and the stimulated specimen's count was 1681 of 2267 or 74.2%. Using CD62 it was 448 of 6053 or 7.4% of the total cells counted and the stimulated specimen's count was 3614 of 4616 or 78.3%. This embodiment for effecting the requisite separation is a preferred embodiment, as one which can also be used to assay for platelet monocyte complexes in a blood specimen.

It is understood that the above discussion with specific examples as well as the drawings are merely illustrative of the present invention and that changes in procedure, materials and processing steps are possible without departing from the scope of the present invention as defined in the following claims.

What is claimed is:

1. A method for adhering cells of interest or a set of cells containing cells of interest as a subset to a limited area of a stationary specimen substrate for examination by a laser scanning cytometer; said method comprising the steps of:

(a) using adhering means with a heterogeneous cell specimen sample to adhere selected cells of interest to a substrate, said adhering means binding to the selected cells of interest or to a selected set of cells including the cells of interest as a subset thereof;

(b) if relevant, discriminating with discrimination means between adhered cells of the subset of interest from other cells of a set if a selected set of cells is adhered; and

(c) analyzing cells of interest with a laser scanning cytometer in the limited area of the substrate.

2. The method of claim 1 wherein said method comprises the steps of:

(a) treating a heterogeneous cell specimen sample with adhering means which bind to cells of interest or to a selected set of cells including the cells of interest as a subset thereof;

(b) adhering cells bound with the adhering means to a limited area substrate by secondary adhering means;

(c) if relevant, discriminating with discrimination means between adhered cells of the subset of interest from other cells of a set if a selected set of cells is adhered; and

(d) analyzing cells of interest with a laser scanning cytometer in the limited area of the substrate.

3. A device for use in effecting the method of claim 1, said device comprising a substrate comprising a fluid specimen loading conduit, and non-specimen flow impeding gating means, comprising secondary adhering means, in a minimal area of the flow path of the conduit, wherein said gating means is adapted to effect adhering thereto of cells having primary adhering

means attached thereto, with application of the secondary adhering means being only a pre-selected limited gating area.

4. A method for efficiently analyzing specific cells of interest with a laser scanning cytometer from a heterogeneous specimen sample, said method comprising the steps of:

a) removing a set of cells, having specific known characteristics, and including a subset of the cells of interest, from a heterogeneous cell specimen sample by adhering at least a significant portion of the set of cells, to a pre-selected limited area of an analysis substrate by adhering means sensitive to the known characteristics;

b) marking only the cells of interest within the significant portion with marking means to identify the subset of cells of interest within the significant portion; and

c) selectively analyzing the identifiable cells of interest within the significant portion, with a laser scanning cytometer, for the determination of at least one characteristic thereof.

5. The method of claim 4, wherein selected marking reagents are used to identify the subset of cells of interest in the significant portion and wherein at least one other reagent is used for reaction with the subset of cells for determination of said at least one characteristic.

6. The method of claim 1, wherein the adhering means comprises an antibody specific to the cells of interest or the set of cells containing cells of interest as a subset, wherein the antibody is coated on said limited area of the substrate.

7. The method of claim 1, wherein the adhering means comprises treating only the cells of interest or the set of cells containing cells of interest as a subset, with magnetic particles coated with an antibody specific to a cell surface antigen of the cells of interest or the set of cells containing cells of interest as a subset and applying a magnetic field gradient to the heterogeneous cell specimen.

8. The method of claim 4, wherein the method is used for detection of activation of blood granulocytes by interaction with bacteria caused by sepsis in newborns.

9. The method of claim 8, for analyzing cells in a blood sample, for detection of activation of blood granulocytes by interaction with bacteria caused by sepsis in newborns, wherein the set of cells comprises leukocytes and the subset of cells of interest comprises granulocytes, said method comprising the steps of:

i) taking a heterogeneous blood sample from the newborn for the testing thereof;

ii) conjugating a fluorescent dye to a CD15 antibody and binding the conjugated fluorescent dye to granulocytes as a marker for the identification of the granulocytes;

iii) conjugating a second fluorescent dye to the antibody CD11b for expressing on the surface of the granulocytes fluorescence as a function of the activation state of the granulocytes;

iv) mixing the two conjugated fluorescent dyes with the specimen;

v) providing the pre-selected limited area of the analysis substrate with a CD45 antibody;

vi) placing the blood sample on the substrate whereby the leukocytes, with contained granulocytes subset, bind and adhere to the CD45 antibody in the pre-selected limited area;

vii) identifying granulocytes as cells of interest with a laser scanning cytometer; and

viii) measuring the activation with a laser scanning cytometer.

10. The method of claim 4, wherein the method is used for detection of activation of blood granulocytes by interaction with bacteria caused by sepsis in adults.

11. The method of claim 10, for analyzing cells in a blood sample, for detection of activation of blood granulocytes by

interaction with bacteria caused by sepsis in adults, wherein the set of cells comprises leukocytes and the subset of cells of interest comprises granulocytes, said method comprising the steps of:

(i) taking a heterogeneous blood sample from the adult for the testing thereof;

(ii) conjugating a fluorescent dye to a CD15 antibody and binding the conjugated fluorescent dye to granulocytes as a marker for the identification of the granulocytes;

(iii) conjugating a second fluorescent dye to the antibody CD64 for expressing on the surface of the granulocytes fluorescence as a function of the activation state of the granulocytes;

iv) mixing the two conjugated fluorescent dyes with the specimen;

v) providing the pre-selected limited area of the analysis substrate with a CD45 antibody;

vi) placing the blood sample on the substrate whereby the leukocytes, with contained granulocytes subset, bind and adhere to the CD45 antibody in the pre-selected limited area;

vii) identifying granulocytes as cells of interest with a laser scanning cytometer; and

viii) measuring the activation state with a laser scanning cytometer.

12. The method of claim 4, wherein the method is used for determination of condition of a patient after angioplasty or stent surgery by determination of activation of blood granulocytes.

13. The method of claim 12, for analyzing cells in a blood sample, for determination of condition of a patient after angioplasty or stent surgery by determination of activation of blood granulocytes, wherein the set of cells comprises leukocytes and the subset of cells of interest comprises granulocytes, said method comprising the steps of:

i) taking a heterogeneous blood sample from the adult for the testing thereof;

ii) conjugating a fluorescent dye to a CD15 antibody and binding the conjugated fluorescent dye to granulocytes as a marker for the identification of the granulocytes;

iii) conjugating a second fluorescent dye to the antibody CD11b for expressing on the surface of the granulocytes fluorescence as a function of the activation state of the granulocytes;

iv) mixing the two conjugated fluorescent dyes with the specimen;

v) identifying granulocytes as cells of interest with a laser scanning cytometer;

vi) providing the pre-selected limited area of the analysis substrate with a CD45 antibody;

vii) placing the blood sample on the substrate whereby the leukocytes, with contained granulocytes subset, bind and adhere to the CD45 antibody in the pre-selected limited area;

viii) identifying granulocytes as cells of interest with a laser scanning cytometer; and

ix) measuring the activation with a laser scanning cytometer.

14. The method of claim 4, wherein the method is used for detection of myocardial infarctions or other vascular pathologies.

15. The method of claim 14, for analyzing cells in a blood sample, for detection of myocardial infarctions or other vascular pathologies, wherein the set of cells comprises leukocytes and the subset of cells of interest comprises monocytes, said method comprising the steps of:

i) taking a heterogeneous blood sample from a patient for the testing thereof;

ii) conjugating a first fluorescent dye to a CD14 antibody for the identification of the monocytes;

iii) conjugating a second fluorescent dye to an antibody which binds to platelets for identifying platelets bound to monocytes;

iv) mixing the two conjugated fluorescent dyes with the specimen;

v) providing the pre-selected limited area of the analysis substrate with a CD45 antibody;

vi) placing the blood sample on the substrate whereby the leukocytes, with contained monocytes, bind and adhere to the CD45 antibody in the pre-selected limited area; and

vii) measuring the fluorescence of the first and second fluorescent dye with a laser scanning cytometer and determining the ratio of number of monocytes with second fluorescence to the total number of monocytes from the first fluorescence as an indication of myocardial infarction or other vascular pathology.

16. The method of claim 4, wherein the method is used for assaying for drug occupancy on a cell surface.

17. The method of claim 16, for analyzing cells in a blood sample, for assaying for drug occupancy on a cell surface, wherein the set of cells comprises platelets and the subset of interest comprises platelet GPIIb/IIIa, said method comprising the steps of:

i) taking a heterogeneous blood sample from a patient, for the testing thereof;

ii) activating the blood sample with an agonist to cause fibrinogen binding to non-drug occupied platelets;

iii) conjugating a first fluorescent dye to an antibody that will bind to GPIIb/IIIa protein;

iv) conjugating a second fluorescent dye to the antibody RIBS for binding to GPIIb/IIIa protein bound to fibrinogen;

v) mixing the two conjugated fluorescent dyes with the specimen;

vi) providing the pre-selected limited area of the analysis substrate with an antibody that will bind to platelets;

vii) placing the blood sample on the substrate whereby the platelets, with contained platelet GPIIb/*IIIa* which may be bound to fibrinogen subset, bind and adhere to the antibody in the pre-selected limited area;

viii) measuring the fluorescence of the first and second fluorescent dyes with a laser scanning cytometer and determining the ratio of the second fluorescence to first fluorescence intensity, with the distribution thereof and computed derivatives providing an assay determination of drug occupancy.

18. The method of claim 4, wherein the method is used for immunophenotyping.

19. The method of claim 18, wherein T4 cells are counted for monitoring of AIDS patients.

20. The method of claim 19, for analyzing cells in a blood sample, for counting of T4 cells for said monitoring, wherein the set of cells comprises leukocytes and the subset of cells of interest comprises T lymphocytes, said method comprising the steps of:

i) taking a heterogeneous blood sample from a patient for the testing thereof;

ii) providing the pre-selected limited area of the analysis substrate with a CD45 antibody;

iii) conjugating a first fluorescent dye to a CD3 antibody as a marker for the identification of the T lymphocytes;

iv) conjugating a second fluorescent dye to the antibody CD4 for binding to T4 helper lymphocytes;

v) mixing the two conjugated fluorescent dyes with the specimen;

vi) placing the blood sample on the substrate whereby the leukocytes bind and adhere to the CD45 antibody in the pre-selected limited area; and

vii) measuring the fluorescence of the first and second fluorescent dye with a laser scanning cytometer and determining the ratio of number of T4 helper lymphocytes with second fluorescence to the total number of T lymphocytes from the first fluorescence.

21. The method of claim 4, for analyzing cells in a blood sample, for detection of activation of blood granulocytes by interaction with bacteria caused by sepsis in newborns, wherein the set of cells comprises leukocytes and the subset of cells of interest comprises granulocytes, said method comprising the steps of:

i) taking a heterogeneous blood sample from the newborn for the testing thereof;

ii) attaching magnetically susceptible particles to a CD45 antibody;

iii) conjugating a fluorescent dye to a CD15 antibody as a marker for the identification of the granulocytes;

iv) conjugating a second fluorescent dye to the antibody CD11b for expressing on the surface of the granulocytes fluorescence as a function of the activation state of the granulocytes;

v) mixing the two conjugated dyes and the antibody CD45, with attached magnetically susceptible particles, with the heterogeneous blood sample whereby the magnetically susceptible particles are bound to leukocytes via the antibody CD45;

vi) thereafter placing the blood sample on the substrate and using magnetic means to cause the leukocytes, with bound magnetically susceptible particles and contained granulocytes subset, to adhere to the pre-selected limited area;

vii) identifying granulocytes as cells of interest with a laser scanning cytometer; and

viii) measuring the activation state with a laser scanning cytometer.

22. The method of claim 4, for analyzing cells in a blood sample, for detection of activation of blood granulocytes by

interaction with bacteria caused by sepsis in adults, wherein the set of cells comprises leukocytes and the subset of cells of interest comprises granulocytes, said method comprising the steps of:

i) taking a heterogeneous blood sample from the adult for the testing thereof;

ii) attaching magnetically susceptible particles to a CD45 antibody;

iii) conjugating a fluorescent dye to a CD15 antibody as a marker for the identification of the granulocytes;

iv) conjugating a second fluorescent dye to the antibody CD64 for expressing on the surface of the granulocytes fluorescence as a function of the activation state of the granulocytes;

v) mixing the two conjugated dyes and the antibody CD45, with attached magnetically susceptible particles, with the heterogeneous blood sample whereby the magnetically susceptible particles are bound to leukocytes via the antibody CD45;

vi) thereafter placing the blood sample on the substrate and using magnetic means to cause the leukocytes, with bound magnetically susceptible particles and contained granulocytes subset, to adhere to the pre-selected limited area;

vii) identifying granulocytes as cells of interest with a laser scanning cytometer; and

viii) measuring the activation state with a laser scanning cytometer.

23. The method of claim 4, for analyzing cells in a blood sample, for determination of condition of a patient after angioplasty or stent surgery by determination of activation of blood granulocytes, wherein the set of cells comprises leukocytes and the subset of cells of interest comprises granulocytes, said method comprising the steps of:

i) taking a heterogeneous blood sample for the testing thereof;

ii) attaching magnetically susceptible particles to a CD45 antibody;

iii) conjugating a fluorescent dye to a CD15 antibody as a marker for the identification of the granulocytes;

iv) conjugating a second fluorescent dye to the antibody CD11b for expressing on the surface of the granulocytes fluorescence as a function of the activation state of the granulocytes;

v) mixing the two conjugated dyes and the antibody CD45, with attached magnetically susceptible particles, with the heterogeneous blood sample whereby the magnetically susceptible particles are bound to leukocytes via the antibody CD45;

vi) thereafter placing the blood sample on the substrate and using magnetic means to cause the leukocytes, with bound magnetically susceptible particles and contained granulocytes subset, to adhere to the pre-selected limited area;

vii) identifying the granulocytes as cells of interest with a laser scanning cytometer; and

viii) measuring the activation state with a laser scanning cytometer.

24. The method of claim 4, for analyzing cells in a blood sample, for detection of myocardial infarctions, wherein the set of cells comprises leukocytes and the subset of cells of interest comprises monocytes, said method comprising the steps of:

i) taking a heterogeneous blood sample from a patient for the testing thereof;

ii) attaching magnetically susceptible particles to a CD45 antibody;

iii) conjugating a first fluorescent dye to a CD14 antibody as a marker for the identification of the monocytes;

iv) conjugating a second fluorescent dye to an antibody which binds to platelets for binding to platelets bound to monocytes;

v) mixing the two conjugated dyes and antibody CD45, with attached magnetically susceptible particles, with the heterogeneous blood sample whereby the magnetically susceptible particles are bound to leukocytes via the antibody CD45;

vi) thereafter placing the blood sample on the substrate and using magnetic means to cause the leukocytes, with bound magnetically susceptible particles and contained monocytes to adhere to the pre-selected limited area; and

vii) measuring the fluorescence of the first and second fluorescent dyes with a laser scanning cytometer and determining the ratio of number of monocytes with second fluorescence to the total number of monocytes from the first fluorescence.

25. The method of claim 20, for analyzing cells in a blood sample, for counting of T4 cells for said monitoring, wherein the set of cells comprises leukocytes and the subset of cells of interest comprises T lymphocytes, said method comprising the steps of:

i) taking a heterogeneous blood sample from a patient for the testing thereof;

ii) attaching magnetically susceptible particles to a CD45 antibody;

iii) conjugating a first fluorescent dye to a CD3 antibody as a marker for the identification of the T lymphocytes;

iv) conjugating a second fluorescent dye to the antibody CD4 used to identify T4 helper lymphocytes;

v) mixing the two conjugated dyes and the antibody CD45, with attached magnetically susceptible particles, with the heterogeneous blood sample whereby the magnetically susceptible particles are bound to leukocytes via the antibody CD45;

vi) thereafter placing the blood sample on the substrate and using magnetic means to cause the leukocytes, with bound magnetically susceptible particles and contained T lymphocytes subset; and

vii) measuring the fluorescence of the first and second fluorescent dyes with a laser scanning cytometer and determining the ratio of number of T4 helper lymphocytes with second fluorescence to the total number of T lymphocytes from the first fluorescence.

26. The method of claim 4, for analyzing cells in a blood sample, for assaying for drug occupancy on a cell surface, wherein the set of cells comprises platelets and the subset of interest comprises platelet GPIIb/IIIa, said method comprising the steps of:

i) taking a heterogeneous blood sample from a patient, after treatment with a drug, for the testing thereof;

ii) activating the blood sample with an agonist to cause fibrinogen binding to non-drug occupied platelets;

iii) attaching magnetically susceptible particles to an antibody that will bind to platelets;

iv) conjugating a first fluorescent dye to an antibody that will bind to GPIIb/IIIa protein;

iv) conjugating a second fluorescent dye to the antibody RIBS for binding to GPIIb/IIIa protein which may be bound to fibrinogen;

v) mixing the two conjugated dyes and the platelet specific antibody, with attached magnetically susceptible particles, with the heterogeneous blood sample whereby the magnetically susceptible particles are bound to platelets, with contained platelet GPIIIb/IIa, which may be bound to fibrinogen, subset;

vi) thereafter placing the blood sample on the substrate and using magnetic means to cause platelets, with contained platelet GPIIIb/IIa bound to fibrinogen subset, to adhere to the pre-selected limited area; and

vii) measuring the fluorescence of the first and second fluorescent dyes with a laser scanning cytometer and determining the ratio of the second fluorescence to first fluorescence intensity, with the distribution thereof and

computed derivatives providing an assay determination of drug occupancy.

27. The method of claim 1, wherein said method is used for analyzing bacteria in a specimen.

28. The method of claim 27, for analyzing bacteria in a specimen wherein the cells of interest are selected from the group consisting of live bacteria and bacteria of specific type, said method comprising the steps of:

i) taking a sample of the specimen for the testing of bacteria content therein;

ii) conjugating lectins to magnetically susceptible particles;

iii) adding a first nucleic acid fluorescent dye to the specimen;

iv) optionally conjugating additional fluorescent dye to bacterial specific antibodies and then adding the additional fluorescent dye, with bacterial specific antibodies, to the specimen to determine the type of specific bacteria as a function of the specific antibodies to which they are conjugated;

v) binding the bacteria to the magnetically susceptible particles, via the lectins;

vi) placing the specimen on a testing substrate and using magnetic means to cause the bacteria to adhere to a pre-selected limited area of the testing substrate;

vii) measuring the fluorescence of the first and optionally second fluorescent dyes with a laser scanning cytometer and determining the number and optionally, type of bacteria by measuring one or more fluorescences with a laser scanning cytometer.

29. The method of claim 27, for analyzing bacteria wherein the cells of interest are live bacteria, said method comprising the steps of:

i) taking a sample of the specimen for the testing of bacteria content therein;

ii) adding lectins conjugated to magnetic particles;

iii) adding a first nucleic acid fluorescent dye;

iv) adding a second fluorescent dye or fluorescent substrate which causes live bacteria to fluoresce;

v) binding the bacteria to the magnetically susceptible particles, via the lectins;

vi) using magnetic means to cause the magnetically susceptible bacteria to adhere to a pre-selected limited area of the testing substrate;

vii) measuring the fluorescence of the first and second fluorescent dyes with a laser scanning cytometer and determining the ratio of number of live and dead bacteria from the first and second fluorescences.

30. The method of claim 27, for analyzing bacteria in a bacteria proliferation medium wherein the cells of interest are live bacteria, said method comprising the steps of:

i) taking a sample for the testing of bacteria content therein;

ii) optionally adding lectins conjugated to magnetically susceptible particles;

iii) adding a fluorescent dye or fluorescent substrate, which causes live bacteria to fluoresce;

iv) placing the sample containing bacteria and growth media on the testing substrate whereby bacteria is adhered to the pre-selected limited area of the testing substrate;

v) measuring the fluorescence of the fluorescent dye with a laser scanning cytometer and determining and recording both the amount of fluorescence and the position of each fluorescent event found; and

vi) repeating the step v at predetermined time intervals to determine the growth of bacteria in said medium.

31. A method for placing and analyzing cells of interest or a set of cells containing cells of interest as a subset on a limited area of a stationary specimen substrate for examination

by a laser scanning cytometer; said method comprising the steps of:

(a) using filtering means with a heterogeneous cell specimen sample to cause selected cells of interest to occupy the surface of a substrate, said filtering means causing the selected cells of interest or a selected set of cells including the cells of interest as a subset thereof to occupy a limited area of a filter surface, with said filter surface comprising the stationary specimen substrate, while passing irrelevant cells through pores in the filter;

(b) if relevant, discriminating with discrimination means between cells of the subset of interest from other cells of a set if a selected set of cells is occupied on the surface of the filter; and

(c) analyzing cells of interest with a laser scanning cytometer in the limited area of the filter surface substrate.

32. A device for use in effecting the method of claim 31, said device comprising a filter substrate comprising a fluid specimen loading conduit, and non-specimen flow impeding gating means, comprising secondary filtering means, in a minimal area of the flow path of the conduit, wherein said gating means is adapted to effect placement thereto of cells having size or deformability differences, with application of the secondary filtering means being only to a pre-selected limited gating area.

33. A method for efficiently analyzing specific cells of interest with a laser scanning cytometer from a heterogeneous specimen sample, said method comprising the steps of:

a) removing a set of cells, having specific known characteristics, and including a subset of the cells of interest, from a heterogeneous cell specimen sample by placing at least a significant portion of the set of cells, on a pre-selected limited area of an analysis substrate utilizing filtering means sensitive to one of the size or deformability of the cells of interest;

b) marking only the cells of interest within the significant portion with marking means to identify the subset of cells of interest within the significant portion; and

c) selectively analyzing the identifiable cells of interest within the significant portion, with a laser scanning cytometer, for the determination of at least one characteristic thereof.

34. The method of claim 33, wherein selected marking reagents are used to identify the subset of cells of interest in the significant portion and wherein at least one other reagent is used for reaction with the subset of cells for determination of said at least one characteristic.

35. The method of claim 33, wherein the method is used for detection of activation of blood granulocytes by interaction with bacteria caused by sepsis in newborns.

36. The method of claim 35, for analyzing cells in a blood sample, for detection of activation of blood granulocytes by interaction with bacteria caused by sepsis in newborns, wherein the set of cells comprises leukocytes and the subset of cells of interest comprises granulocytes, said method comprising the steps of:

i) taking a heterogeneous blood sample from the newborn for the testing thereof;

ii) conjugating a fluorescent dye to a CD15 antibody and binding the conjugated fluorescent dye to granulocytes as a marker for the identification of the granulocytes;

iii) conjugating a second fluorescent dye to the antibody CD11b for expressing on the surface of the granulocytes fluorescence as a function of the activation state of the granulocytes;

iv) mixing the two conjugated fluorescent dyes with the specimen;

v) placing the blood sample on the substrate whereby the leukocytes, with contained granulocytes subset, are placed in the pre-selected limited area;

vi) identifying granulocytes as cells of interest with

a laser scanning cytometer; and

vii) measuring the activation with a laser scanning cytometer.

37. The method of claim 33, wherein the method is used for detection of activation of blood granulocytes by interaction with bacteria caused by sepsis in adults.

38. The method of claim 37, for analyzing cells in a blood sample, for detection of activation of blood granulocytes by interaction with bacteria caused by sepsis in adults, wherein the set of cells comprises leukocytes and the subset of cells of interest comprises granulocytes, said method comprising the steps of:

(i) taking a heterogeneous blood sample from the adult for the testing thereof;

(ii) conjugating a fluorescent dye to a CD15 antibody and binding the conjugated fluorescent dye to granulocytes as a marker for the identification of the granulocytes;

(iii) conjugating a second fluorescent dye to the antibody CD64 for expressing on the surface of the granulocytes fluorescence as a function of the activation state of the granulocytes;

iv) mixing the two conjugated fluorescent dyes with the specimen;

v) placing the blood sample on the substrate whereby the leukocytes, with contained granulocytes subset, are placed in the pre-selected limited area;

vi) identifying granulocytes as cells of interest with a laser scanning cytometer; and

vii) measuring the activation state with a laser scanning cytometer.

39. The method of claim 33, wherein the method is used for determination of condition of a patient after angioplasty or stent surgery by determination of activation of blood granulocytes.

40. The method of claim 39, for analyzing cells in a blood sample, for determination of condition of a patient after angioplasty or stent surgery by determination of activation of blood granulocytes, wherein the set of cells comprises leukocytes and the subset of cells of interest comprises granulocytes, said method comprising the steps of:

i) taking a heterogeneous blood sample from the adult for the testing thereof;

ii) conjugating a fluorescent dye to a CD15 antibody and binding the conjugated fluorescent dye to granulocytes as a marker for the identification of the granulocytes;

iii) conjugating a second fluorescent dye to the antibody CD11b for expressing on the surface of the granulocytes fluorescence as a function of the activation state of the granulocytes;

iv) mixing the two conjugated fluorescent dyes with the specimen;

v) placing the blood sample on the substrate whereby the leukocytes, with contained granulocytes subset, are placed in the pre-selected limited area;

vi) identifying granulocytes as cells of interest with a laser scanning cytometer; and

vii) measuring the activation with a laser scanning cytometer.

41. The method of claim 33, wherein the method is used for detection of myocardial infarctions or other vascular pathologies.

42. The method of claim 41, for analyzing cells in a blood sample, for detection of myocardial infarctions or other vascular pathologies, wherein the set of cells comprises leukocytes and the subset of cells of interest comprises monocytes, said method comprising the steps of:

i) taking a heterogeneous blood sample from a patient for the testing thereof;

ii) conjugating a first fluorescent dye to a CD14 antibody for the identification of the monocytes;

iii) conjugating a second fluorescent dye to an antibody which binds to platelets for identifying platelets bound to monocytes;

iv) mixing the two conjugated fluorescent dyes with the specimen;

v) placing the blood sample on the substrate whereby the leukocytes, with contained monocyte subset, are placed in the pre-selected limited area;

vi) measuring the fluorescence of the first and second fluorescent dye with a laser scanning cytometer and determining the ratio of number of monocytes with second fluorescence to the total number of monocytes from the first fluorescence as an indication of myocardial infarction or other vascular pathology.

43. The method of claim 33, wherein the method is used for immunophenotyping.

44. The method of claim 33, wherein T4 cells are counted for monitoring of AIDS patients.

45. The method of claim 44, for analyzing cells in a blood sample, for counting of T4 cells for said monitoring, wherein the set of cells comprises leukocytes and the subset of cells of interest comprises T lymphocytes, said method comprising the steps of:

i) taking a heterogeneous blood sample from a patient for the testing thereof;

ii) conjugating a first fluorescent dye to a CD3 antibody as a marker for the identification of the T lymphocytes;

iii) conjugating a second fluorescent dye to the antibody CD4 for binding to T4 helper lymphocytes;

iv) mixing the two conjugated fluorescent dyes with the specimen;

v) placing the blood sample on the substrate whereby the leukocytes, with contained lymphocyte subset, are placed in the pre-selected limited area;

vi) measuring the fluorescence of the first and second fluorescent dye with a laser scanning cytometer and determining the ratio of number of T4 helper lymphocytes with second fluorescence to the total number of T lymphocytes from the first fluorescence.

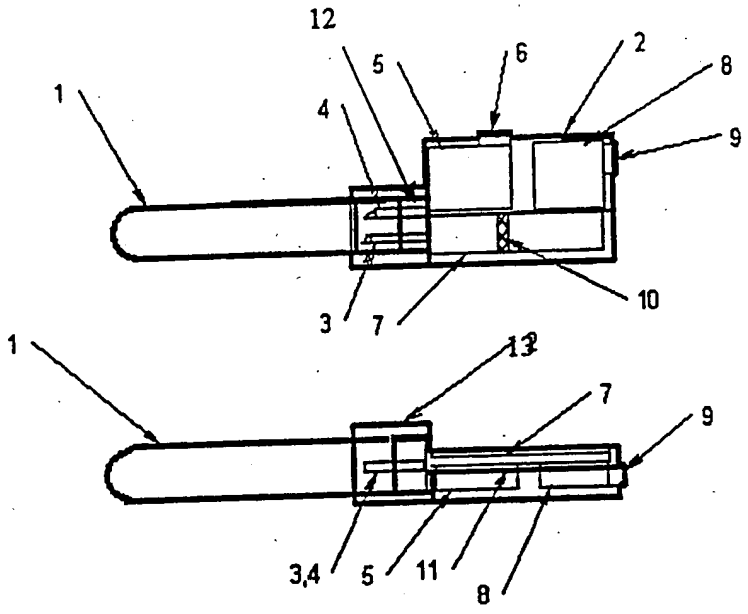


Fig 1a

FIG. 1b

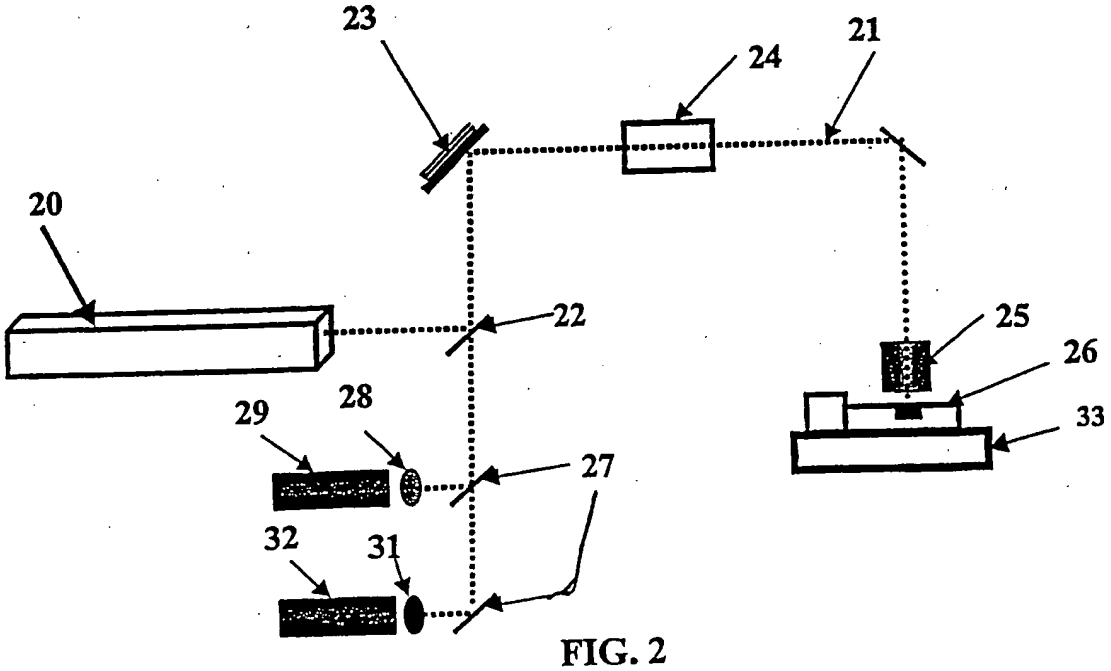


FIG. 2

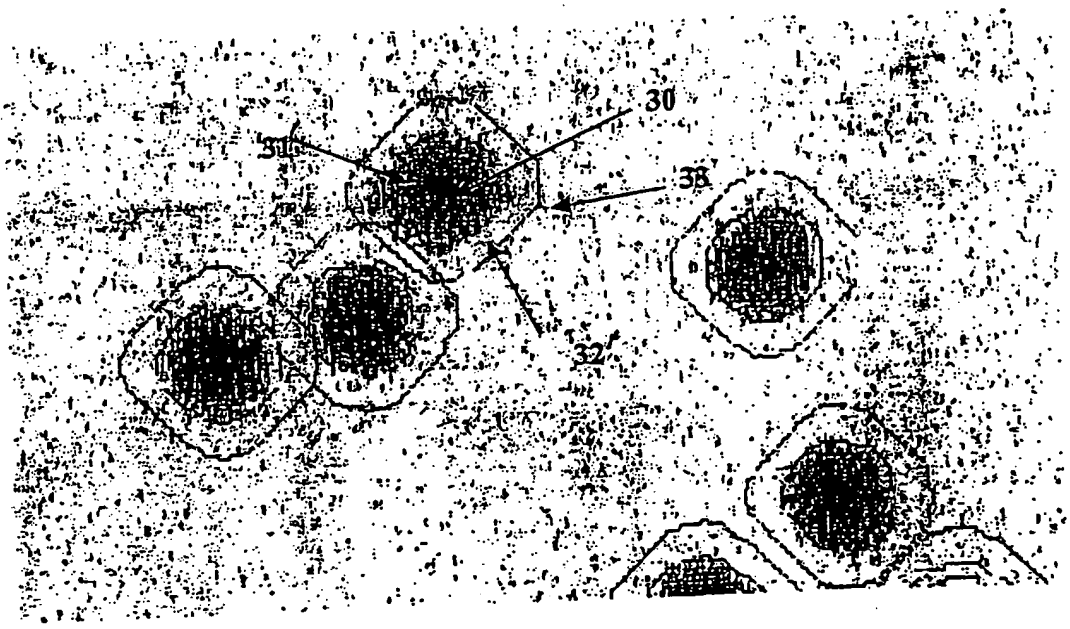


FIG. 3

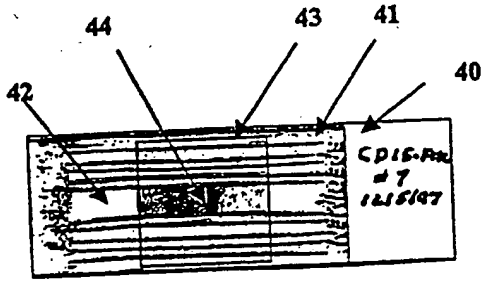
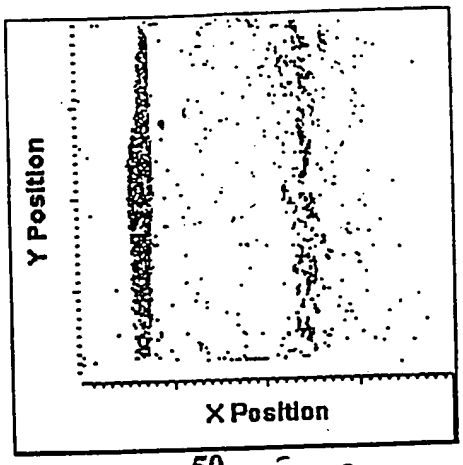
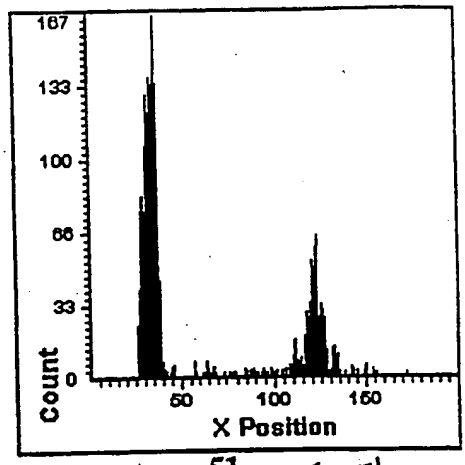


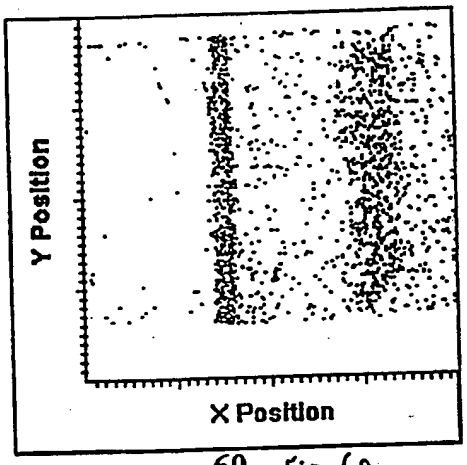
FIG. 4



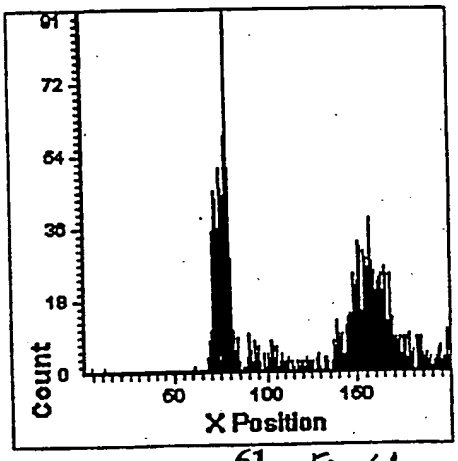
50 Fig 5a



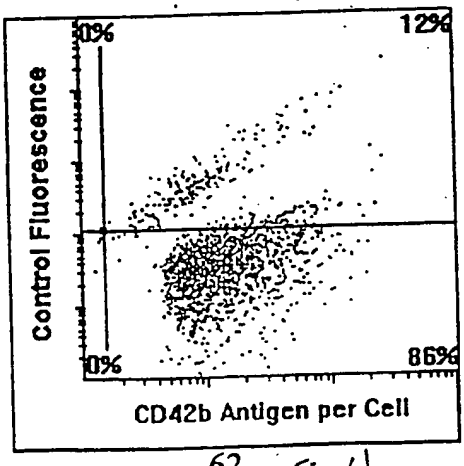
51 Fig 5b



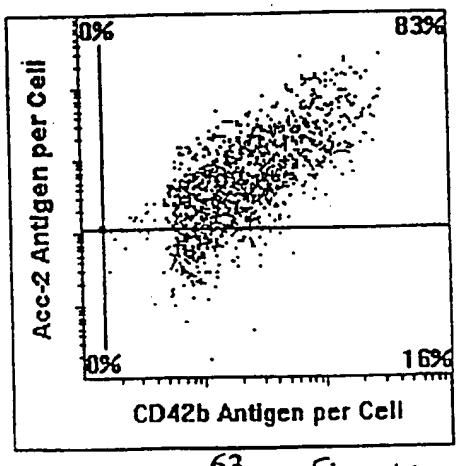
60 Fig 6a



61 Fig 6d



62 Fig 6b



63 Fig 6c

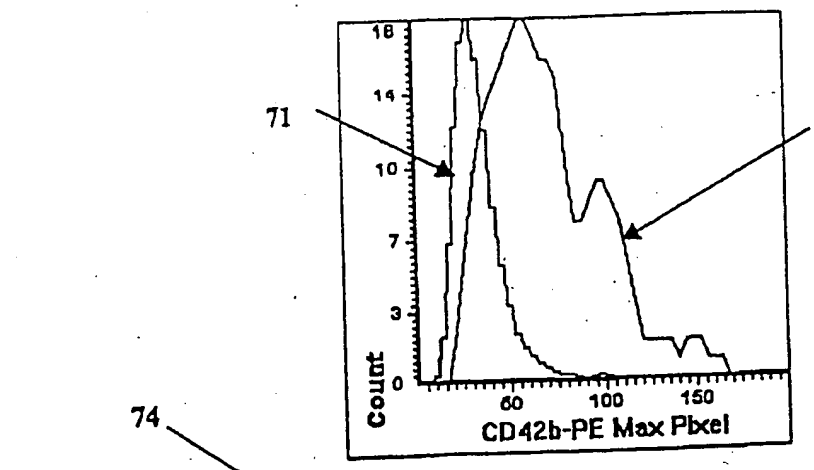


FIG 7a

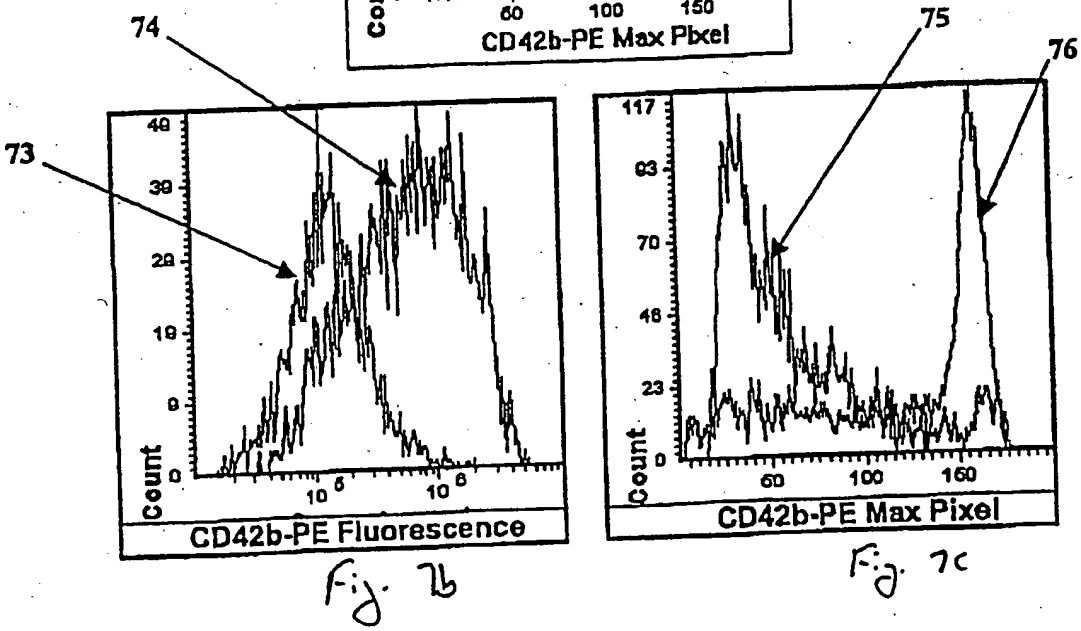


Fig. 7b

Fig. 7c

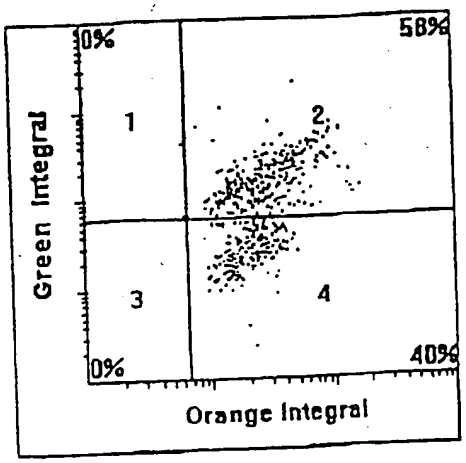
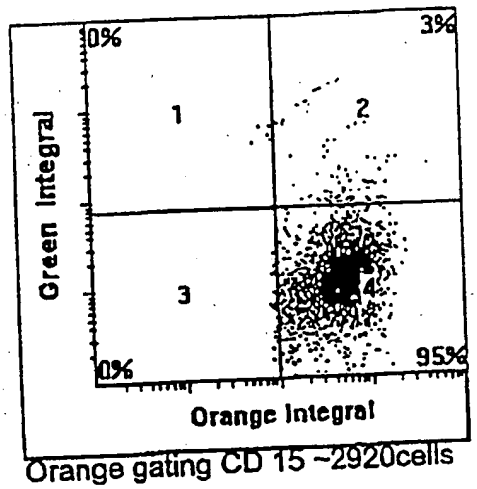
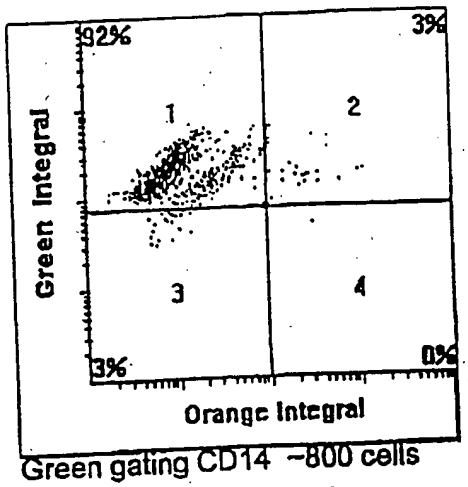


FIG. 8



Orange gating CD 15 ~2920cells

Fig 9a



Green gating CD14 ~800 cells

Fig 9b

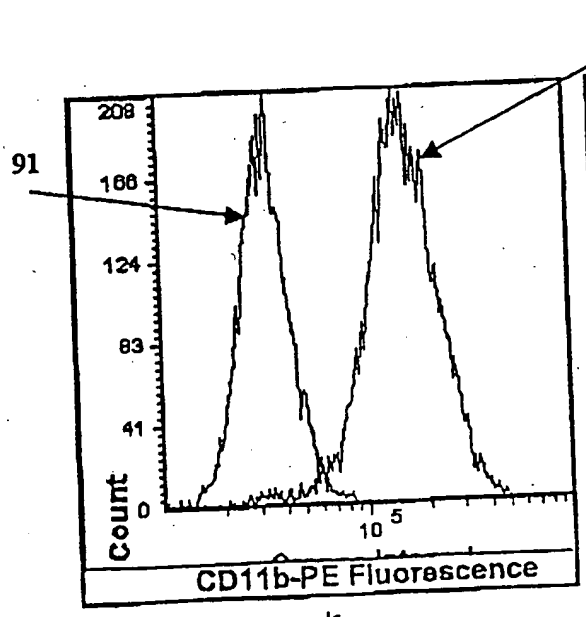


Fig 10a

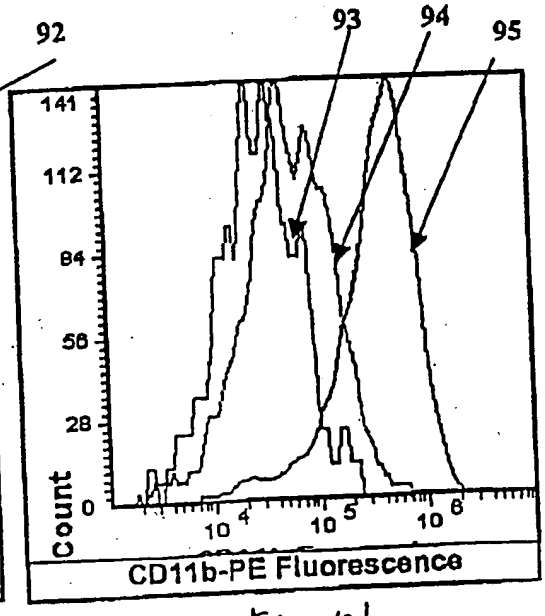


Fig 10b

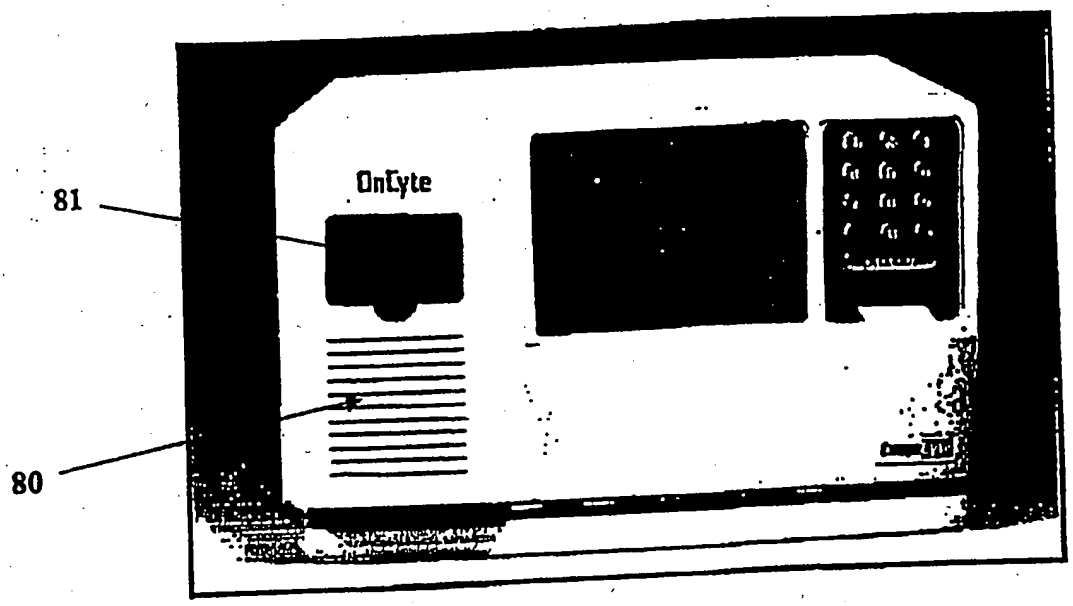


FIG. 11

Fig 12b

Fig. 12a

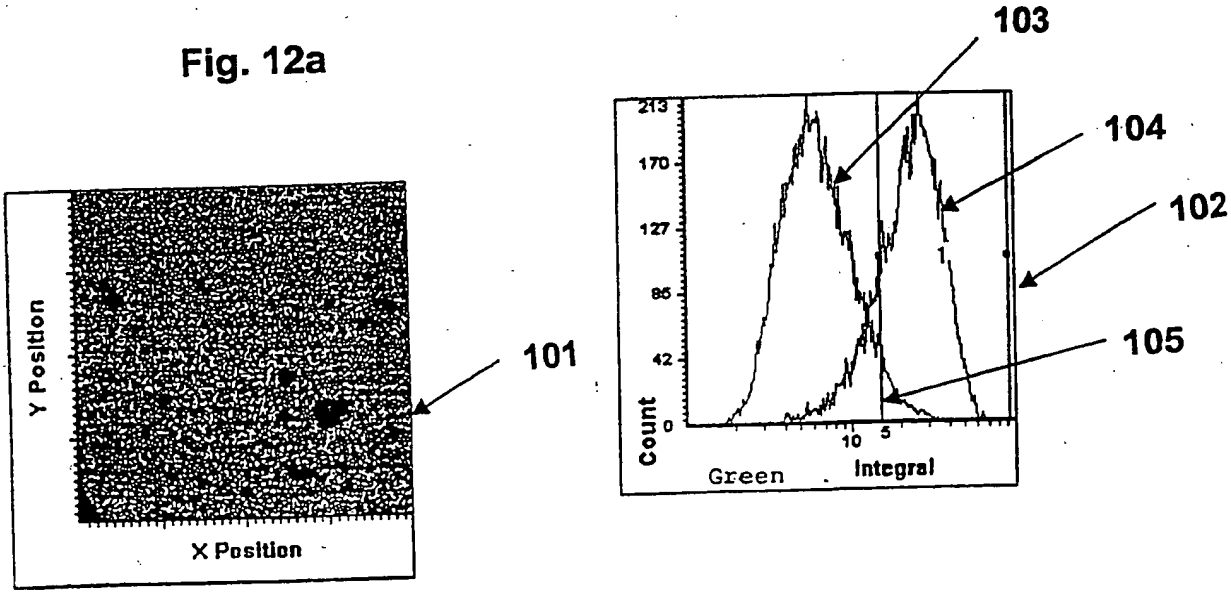
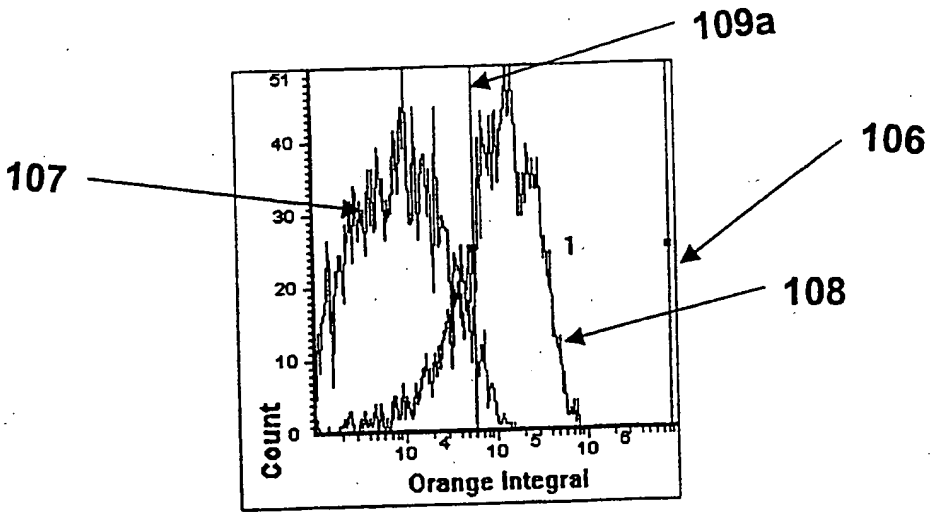


Fig. 13a



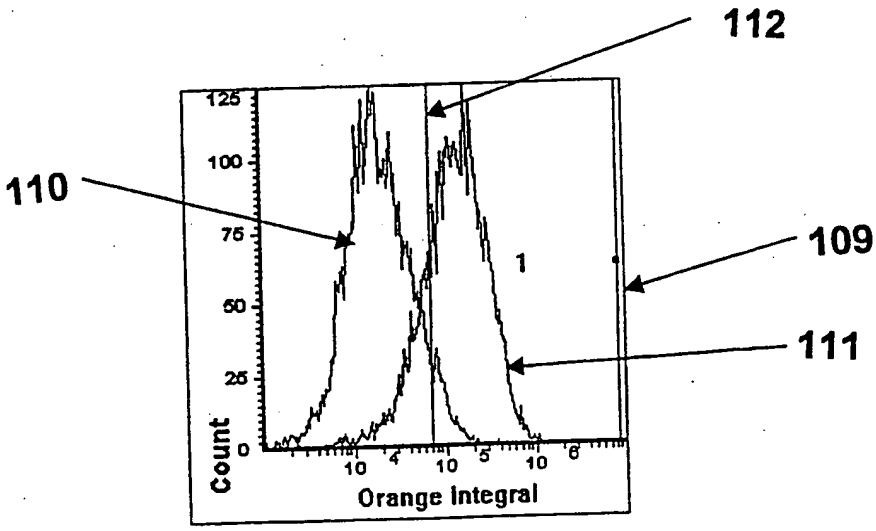


Fig. 13b

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/04415

A. CLASSIFICATION OF SUBJECT MATTER
IPC(6) :C12M 1/00, 1/12; C12Q 1/00, 1/06, 1/24, 1/68; G01N 33/543, 33/569
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/4, 6, 7.2, 7.21, 7.24, 7.32, 30, 39, 287.2, 287.7, 287.8, 287.9, 288.5, 288.7; 436/63, 526

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,053,344 A (ZBOROWSKI et al) 01 October 1991. See abstract; see Figures 1 and 2; see column 6, line 52-column 7, line 2.	3
A	US 5,100,777 A (CHANG) 31 March 1992. See abstract; see column 4, lines 27-31 and 43-61.	1-6, 8-20
A	US 5,107,422 A (KAMENTSKY et al) 21 April 1992. See abstract.	1-45
A	US 5,602,042 A (FARBER) 11 February 1997. See column 10, lines 10-44.	1-5, 7, 21-30
X — A	US 5,660,990 A (RAO ET AL) 26 August 1997. See column 5, line 5-column 7, line 30; column 9, lines 30-65.	3 — 1-2, 4-5, 7, 21-30

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
24 MAY 1999	11 JUN 1999

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	DAVID SAUNDERS JOYCE BRIDGERS PARALEGAL SPECIALIST CHEMICAL MATRIX
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/04415

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y ---- A	US 5,663,057 A (DROCOURT et al) 02 September 1997. See abstract; see column 1, lines 41-50; column 3, lines 1-24.	31-34,43 -------- 35-42, 44-45
Y ---- A	KAMENTSKY et al. Slide-based laser scanning cytometry. Acta Cytologica, January-February 1997, Volumn 41, Number 1, pages 123-143. See page 123, column 2-page 125, column 2.	31-34, 43 ---------- 35-42, 44-45

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/04415

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

435/4, 6, 7.2, 7.21, 7.24, 7.32, 30, 39, 287.2, 287.7, 287.8, 287.9, 288.5, 288.7; 436/63, 526